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Phospho-ΔNp63α-responsive microRNAs contribute to the regulation of necroptosis in squamous cell carcinoma upon cisplatin exposure

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ABSTRACT

This study shows that specific microRNAs differentially regulated by ΔNp63α in cisplatin-sensitive and resistant squamous cell carcinoma (SSC) cells of larynx and tongue affect the expression of members of the necroptotic pathway CYLD, RIPK1, and MLKL. Different degrees of protein interaction between necroptotic signaling intermediates were also observed in SCC cells sensitive or resistant to cisplatin. Modulation of RIPK1 with miR-101-3p mimic or inhibitor, as well as with siRNA, or chemical inhibitors was shown to affect sensitivity of SCC cells to cisplatin. This is the first report showing the modulatory effect of ΔNp63α-responsive microRNAs on the specific members of necroptotic pathway in SCC tumor cells variably responding to platinum chemotherapy.

1. Introduction

Maintaining a tight balance between cell survival and cell death is pivotal for various normal and pathological conditions [1,2]. Molecular mechanisms underlying the processes leading to cell death are evolutionarily conserved across species, ranging from yeast, flies, and worms to mammals and humans, supporting the importance of these cellular mechanisms for multicellular organisms [1,2]. A main goal of the anti-cancer chemotherapy is to induce cell death phenotype in tumor cells and to stimulate the series of molecular events altering the survival of tumor cell up to their demise [2–4]. Cellular responses to the stress factors often inducing DNA damage, oxidative overload, and malfunctions in cell survival involve multiple signaling pathways [2,3]. These genetically programmed pathways include cell cycle arrest, apoptosis, autophagy and necroptosis among others [2–6]. Several proteins involved in apoptosis or autophagy were shown to participate in a crosstalk with necroptotic molecular machinery [5–7].

Cisplatin is the most used chemotherapeutic agent to treat human head and neck cancer because of its ability to induce cell death, however, tumor cells often developed an acquired resistance to cisplatin [8]. p53 family members (p53, p63 and p73) are key transcriptional factors that orchestrate the cellular response to cisplatin treatment [9–12]. Being at the helm of the transcriptional regulation, p53 family members actively influence the levels of various mRNAs, and subsequently the proteins engaged in multiple processes of cell death (cell arrest, apoptosis, and autophagy), especially during tumorigenesis [9–12].

P53 homologue p63 was shown to directly induce the transcription of CD95 death receptor (FAS, TNFRSF6), which plays a critical role in tumor necrosis factor (TNF)-dependent activation of necroptotic signaling and extrinsic apoptotic signaling [13]. P63-dependent transcriptional regulation of the TNF and TNF-related apoptosis-inducing ligand (TRAIL) death receptor's expression further supports a link between mitochondrial apoptoptic and necroptotic pathways [13,14].

Abbreviations: ATG, autophagy-related protein; ATM, ataxia telangiectasia mutated; BCL, B-cell lymphoma; CIS, cisplatin; CYLD, cylindromatosis; DAP3, death-assocated protein; DAPK, death-associated protein kinase; IAP, inhibitor of apoptosis; IP, immunoprecipitation; MLKL, mixed lineage kinase domain-like; NEC, necrostatin; NSA, necrosulfonamide; p, phosphorylated; RIPK, receptor interacting protein kinase; SCC, squamous cell carcinoma; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAIL, TNF-related apoptosis-inducing ligand; TRADD, tumor necrosis factor receptor type 1-associated death domain protein; TRAF, TNF receptor associated factor; UTR, untranslated region

Author contributions: E.A.R. has designed, performed the experiments, analyzed data and written the manuscript.

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P63 gene encodes at least six different protein isoforms, which consist of two groups, including those with the transactivation (TA)-domain at the amino terminus (TAp63α, TAp63β, and TAp63γ) or those without it (ΔNp63α, ΔNp63β, and ΔNp63γ) [11,12]. Cisplatin treatment was shown to induce the phosphorylation of the predominant p63 isoform, ΔNp63α, in cisplatin-sensitive squamous cell carcinoma cells (SCC-11) by ataxia telangiectasia mutated (ATM) kinase at the position S385 (SCC-11M) [15]. We generated the isogeneic SCC cell lines derived from larynx and expressing the wild type ΔNp63α (SCC11 cells) or mutated ΔNp63α (SCC-11M cells with the altered ability to maintain the phosphorylation of ΔNp63α by ATM kinase) [15–17]. Previous studies have pointed-out that cisplatin-sensitive SCC cells express exclusively (SCC-11, larynx) or predominantly (SCC-25, tongue) a phosphorylated (p)-ΔNp63α protein [18,19]. We further found that these cell lines differentially express specific microRNAs [16–18]. We showed that the specific ΔNp63α-responsive microRNAs are likely to module protein targets involved in cell cycle arrest, apoptosis or autophagy, and epigenetic regulation [16–19]. In this report, we would like to emphasize the role for ΔNp63α-responsive microRNAs in modulation of members of necroptotic signaling pathway suggesting that this new genetically programmed molecular mechanism may contribute to a tumor cell response to cisplatin exposure.

2. Materials and methods

2.1. Cells, reagents and antibodies

Larynx-derived SCC-11 cell line (JHU-011 cell line, formerly known as JHU-029 cell line, expressing wild type p53, wild type p63 is amplified and ΔNp63α is overexpressed) was isolated from primary tissue at the Head and Neck Cancer Research (HNCR) Division (Johns Hopkins Medical Institutions (JHMI), and obtained from the HNCR Tissue Bank as a gift by Dr. Joseph A. Califano. Using the knock-in technology we generated cisplatin-sensitive SCC-11 cells (expressing wild type ΔNp63α protein) and cisplatin-resistant SCC-11M cells (expressing ΔNp63α-S385G protein with an impaired site for ATM kinase phosphorylation) [15]. Tongue-derived SCC-25 cells (sensitive to cisplatin) and SCC-25CP cells (with a spontaneously acquired cisplatin resistance) were obtained from Dr. J.S. Lazo, as reviewed in [18,20].

Cells were incubated with control medium or 10 μg/ml cisplatin (P4394, Sigma–Aldrich). To inhibit RIPK1 and mixed lineage kinase domain-like (MLKL) activities, we used Necrostatin-1 (NEC-1, N9037, Sigma–Aldrich, 30 μM, 1 h, serum-free media) and necrosulfonamide (NSA, C6327-2s, Cellagen Technology, 1 μM, 16 h), respectively. We used antibodies against CASP8 (C7849, 1:1000, Sigma–Aldrich), cylindromatosis (CYLD) (PAI-41097, 1:250), death-assocated protein (DAP3) (MA1-41279, 1:150), and DAPK2 (PA5-19961, 1:500), all obtained from Thermo Scientific-Pierce. We also used antibodies against α-tubulin (ab7291, 1:5000), RIPK3 (ab56164, 1:500), MLKL (ab172868, 1:1000), and p-S358-MLKL (ab187091, 1:1000), all purchased from Abcam, and finally antibody against RIPK1 (NB100-56160, 1:1000, Novus Biologicals). Goat anti-rabbit (AP307P), and goat anti-mouse (AP181P) horseradish peroxidase-conjugated immunoglobulins (IgG) were purchased from EMD-Millipore, and diluted at 1:5000–1:10000].

2.2. Immunoblotting and immunoprecipitation

Cells were lysed in buffer A (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.5% Brij-50, 1 mM PMSF, 4 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM Na_3VO_4, 50 mM NaF), sonicated five times for 10 s, and clarified for 30 min at 15000×*g* [21]. Total lysate supernatants were resolved by a 4–10% SDS–PAGE and analyzed by immunoblotting after transfer onto PVDF membranes overnight. Membranes were blocked with 10% fat-free milk with 0.1% Tween-20 in Tris-buffered saline (TBS) and incubated with primary antibodies for 1 h in 3% fat-free milk, 0.1% Tween-20 in TBS and after washing were incubated with secondary antibodies for an additional 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence kit with the SuperSignal West Pico Chemiluminescent Substrate (Pierce-Thermo Scientific). For immunoprecipitation, total lysate supernatants were pre-incubated with 10 μl of normal rabbit serum (R9133, Sigma) or normal mouse serum (M5905, Sigma) for 30 min and then incubated with 50 μl of protein A-Sepharose 4B for an additional 30 min. Following centrifugation, 500 μl of pre-cleared supernatant was incubated with 10 μg of primary antibodies for 2 h at room temperature and then with 40 μl of a 50% suspension of goat anti-rabbit IgG-bound agarose (ab97052, Abcam) or goat anti-mouse IgG-bound agarose (ab97025, Abcam) overnight at 4 °C, and then was washed three times with 1 ml of cold buffer A. Resulting samples were boiled with SDS and β-mercaptoethanol, fractionated by a SDS–PAGE and transferred onto PVDF membranes for immunoblotting, as mentioned above [21]. Blots were scanned and quantified by the Image Quant software version 3.3 (Molecular Dynamics). Values were normalized for the α-tubulin levels and expressed as percentage of a control sample (defined as 1).

2.3. Transfections and luciferase reporter assays

For microRNA/3′-UTR luciferase reporter assays, we used the control (empty) pLightSwitch_3UTR vector (S890005), the 3′-UTR luciferase reporter plasmids for CASP8 (S808083), MLKL (S805840), DAP3 (S802569), DAPK2 (S809711), RIPK1 (S810475), all were obtained from SwitchGear Genomics (SGG). The 3′-UTR reporter plasmid for CYLD (MiUTR1H-02603) was from Creative Biogene. Cells (40–50% confluency) in a 24-well plate were transfected with the selected 3′-UTR plasmids (0.1 μg), and RenSP Renilla luciferase plasmid (0.01 μg) using a Fugene HD reagent (Roche) for 36 h according to the manufacturer's (SGG) recommendations. Cells were also transfected for 36 h with the following human mirVana® mimics (at a final concentration 50 nM): hsa-miR-485-5p (MC10837), hsa-miR-221-3p (MC10337), hsa-miR-214-3p (MC12124), hsa-miR-155-5p (MC12601), hsa-miR-22-3p (MC10203), hsa-miR-101-3p (MC11414), and inhibitor for hsa-miR-101-3p (AM11414), as well as with the scrambled RNA (mirVana™ miRNA Mimic, Negative Control #1, 4464061, or mirVana™ miRNA Inhibitor, Negative Control #1, 4464078), all purchased from Ambion/Life Technologies.

Transfection efficiency was validated using the gWiz High-Expression GFP vector (P040400, Genlantis), as recommended by Roche. Resulting cells were treated with control media or 10 μg/ml cisplatin for an additional 16 h. The LightSwitch Luciferase Assay Kit (SwitchGear Genomics) was used to monitor a RenSP Renilla luciferase reporter activity at 480 nm using a luminometer. A total GFP fluorescence was measured (linear curve was generated with the increasing concentrations of the GFP plasmid) using a fluorescence plate reader. Luciferase activity of each sample was measured and corrected for total GFP. Each experiment was performed 3 times in triplicate. Data presented as relative values (RU) to data obtained from the control samples (cells transfected with the scrambled RNA and exposed to control media), which designated as 1.

To generate the mutated 3′-UTR plasmids with altered microRNA 'target' sequences, we used a QuikChange Site-

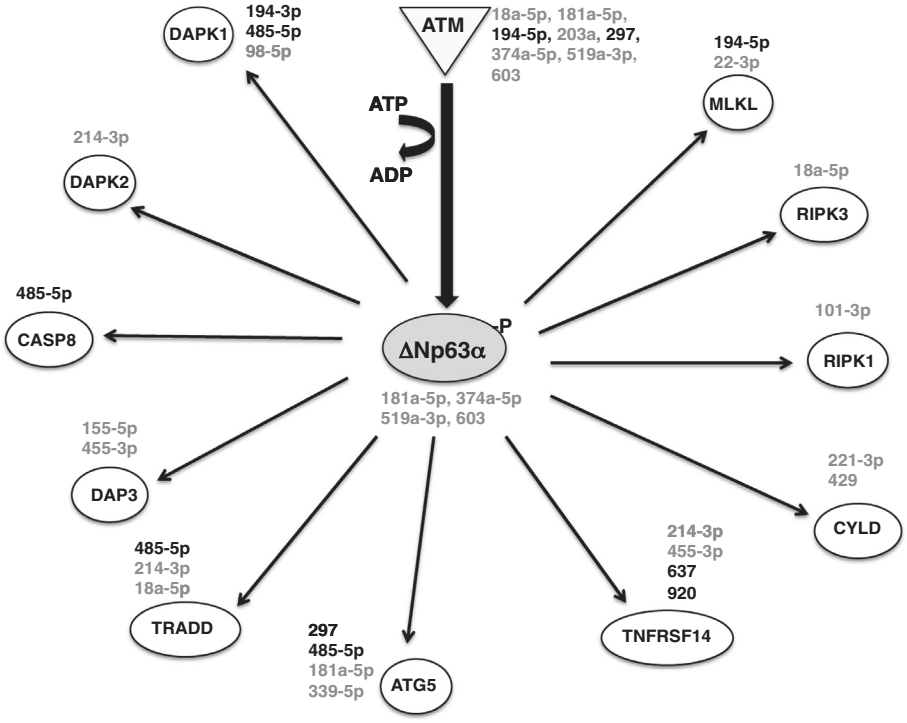

Fig. 1. Schematic representation of the microRNA-regulated targets involved in necroptotic pathway. The microRNAs induced by p-ΔNp63α (SCC-11 vs. SCC-11M) indicated in black, while the repressed microRNAs indicated in gray (SCC-11 vs. SCC-11M) defined by the MicroRNA Target Prediction And Functional Study Databases (see Supplemental info). The corresponding microRNAs are shown next to the specific protein target. MicroRNAs listed next to ATM or ΔNp63α are shown to be inhibitors of ATM or ΔNp63α.

Directed Mutagenesis Kit (Agilent) using 100 ng of each DNA template and 125 ng of each primer (see Supplemental methods). All mutated plasmids were verified by sequencing at the Johns Hopkins Synthesis and Sequencing core facility to confirm the presence of mutations.

In other experiments, cells (40–50% confluency)/in 6-well plate were also transfected with the 20 nM (final concentration) of the scrambled siRNA (Silencer® Negative Control No. 1 siRNA, AM4636) and RIPK1 siRNA (Silencer, #137228-137230, Life Technologies) using 5–10 μl of Lipofectamine-2000 reagent (Invitrogen/Life Technologies) for 36 h. Transfection efficiency was validated using a Silencer® Cy™ 3-labeled Negative Control No. 1 siRNA (AM4621, Life Technologies), as recommended by a manufacturer. Post-transfection, cells were exposed to control medium and 10 μg/ml cisplatin for an additional 16 h. Each experiment was performed 3 times in triplicate.

2.4. Cell viability assay

Cells/in 96-well plates were incubated in serum-free medium with 5 μg/ml of the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay, American Tissue Culture Collection, ATCC 30-1010K) in the dark at 37 °C for 4 h. Lysed cells were incubated at 37 °C for 2 h and the measurements (A_{570} nm to A_{650} nm) were obtained on a Spectra Max 250 plate reader (Molecular Devices). Each assay was repeated three times in triplicate.

2.5. Statistical analysis

Difference between two groups or more than two groups was performed by Student's *t*-test, and one-way ANOVA test. The levels of significance were set to $P \leqslant 0.05$.

3. Results

Based on microRNA chip analysis [16,18] and using the web-based microRNA target prediction databases (see Supplemental methods), we defined *in silico* targets for specific microRNAs down-regulated (marked in gray) and upregulated (marked in black) in SCC-11 cells compared to SCC-11M cells (Fig. 1). Some of these predicted protein targets are implicated in regulation of necroptotic program of tumor cell death [1–3]. Using the 3′-UTR-mediated luciferase activity assay and immunoblotting analysis, we showed that the tested microRNA mimics (e.g. miR-48-5p, miR-221-3p, miR-101-3p, miR-22-3p, miR-155-5p, and miR-214-3p) markedly (by 54–62%) modulated the luciferase reporter activities driven by certain mRNA 3′-UTR plasmids (Fig. 2A). However, these microRNA mimics failed to inhibit the luciferase activities driven by the 3′-UTR plasmids whose microRNA 'target' sequences were mutated (Fig. 2A, Table S1, Supplemental methods). Moreover, tested microRNA mimics variably modulated the levels of CASP8, CYLD, RIPK1, MLKL, DAP3, and DAPK2 proteins involved in necroptotic pathway (Fig. 2B).

We further found that the levels of endogenous CYLD, RIPK1, MLKL, and DAP3 proteins were markedly higher in cisplatin-treated sensitive SCC-11 cells and SCC-25 cells than in cisplatin-treated resistant SCC-11M cells and SCC-25CP cells, respectively (Fig. 3A). However, the CASP8 protein levels in SCC-11/SCC-25 cells was lower than in SCC-11M/SCC-25CP cells (Fig. 3A). We then examined the ability of RIPK1, RIPK3 and p-MLKL to form protein complexes in sensitive (SCC-11 and SCC-25) cells and resistant (SCC-11M and SCC-25CP) cells upon cisplatin exposure. Intriguingly, we observed that the formation of RIPK1/RIPK3/p-MLKL protein complexes occurred in sensitive SCC-11 and SCC-25 cells, while it markedly decreased in resistant SCC-11M and SCC-25CP cells (Fig. 3B).

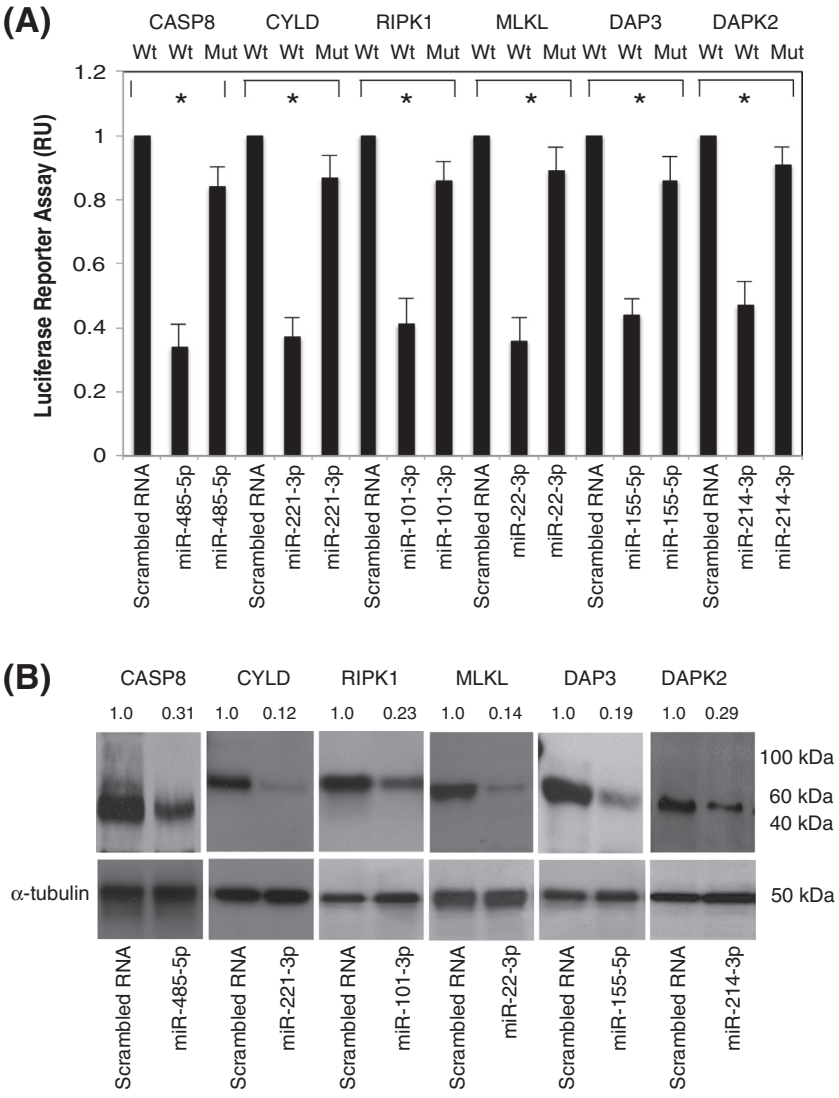

Fig. 2. ΔNp63α-dependent microRNA modulate their mRNA targets in vitro. (A) MicroRNA/3′-UTR luciferase reporter assays for indicated targets in SCC-11 cells exposed to control medium. Target protein symbols are indicated above the graph, while specific microRNA mimics are indicated below the graph. As a negative control, scrambled RNA was used. All indicated 3′-UTR plasmids were also mutated in the microRNA 'target' sites (as described in Supplemental methods and Table S1), and designated as Mut. Experiments were run three times in triplicates and compared to data obtained with SCC-11 cells transfected with the scrambled RNA (*, $P < 0.05$). (B) Immunoblotting with the indicated antibodies. Each blot was first probed with indicated antibodies and after stripping with an antibody to α-tubulin used as a loading control. Lines between blots indicate the separate gel runs. Blots were scanned and quantified in triplicate. Values (shown above the blots) normalized by α-tubulin levels were expressed as a fold change to a scrambled control defined as 1.

The sensitive cells (SCC-11 and SCC-25) were shown to express miR-101-3p, a potential regulator of RIPK1, at the lower level than resistant cells (SCC-11M and SCC-25CP) upon cisplatin exposure [16,18]. We examined whether the inactivation of the key regulator of necroptotic signaling RIPK1 using miR-101-3p mimic and RIPK1 siRNA would affect a viability of sensitive SCC-11 cells and SCC-25 cells. In addition we examined whether the miR-101-3p inhibitor would affect a viability of resistant SCC-11M cells and SCC-25CP cells. We thus observed that both SCC-11 cells and SCC-25 cells were shown to markedly decrease their viability upon cisplatin exposure compared to control media treatment (Fig. 4). Both miR-101-3p mimic and RIPK1 siRNA partially increased the resistance of sensitive SCC cells to cisplatin treatment (Fig. 4). Inhibitory effect of miR-101-3p and RIPK1 siRNA on the RIPK1 protein levels in SCC cells was shown in Fig. S1A. Similarly, the necrostatin (NEC-1, inhibitor of RIPK1) and necrosulfonamide (NSA, MLKL inhibitor) increased the viability of SCC-11 cells and SCC-25 cells upon cisplatin exposure (Fig. 4) suggesting a potential role for a necroptotic pathway in the SCC-11/SCC-25 cell response to cisplatin treatment. The RIPK1 protein levels were not affected by the treatment of SCC cells with NEC-1 and NSA (Fig. S1B). However, the miR-101-3p inhibitor increased the RIPK1 level in resistant SCC-11M cells and SCC-25CP cells (Fig. S1C), and sensitivity of SCC-11M cells and SCC-25CP cells to cisplatin exposure (Fig. 4).

4. Discussion

The exposure of tumor cells to anti-cancer chemotherapeutic compounds was shown to induce DNA damage signaling subsequently leading to cell cycle arrest, apoptosis, autophagy and necroptosis ultimately resulting in tumor cell death [1–5]. However, these treatments are often result in tumor cell resistance, which involve multiple molecular mechanisms ranging from epigenetic deregulation, alterations in apoptosis and autophagy and even metabolic changes between tumor cells sensitive and resistant to acquire cell death phenotype [1–5,8–14].

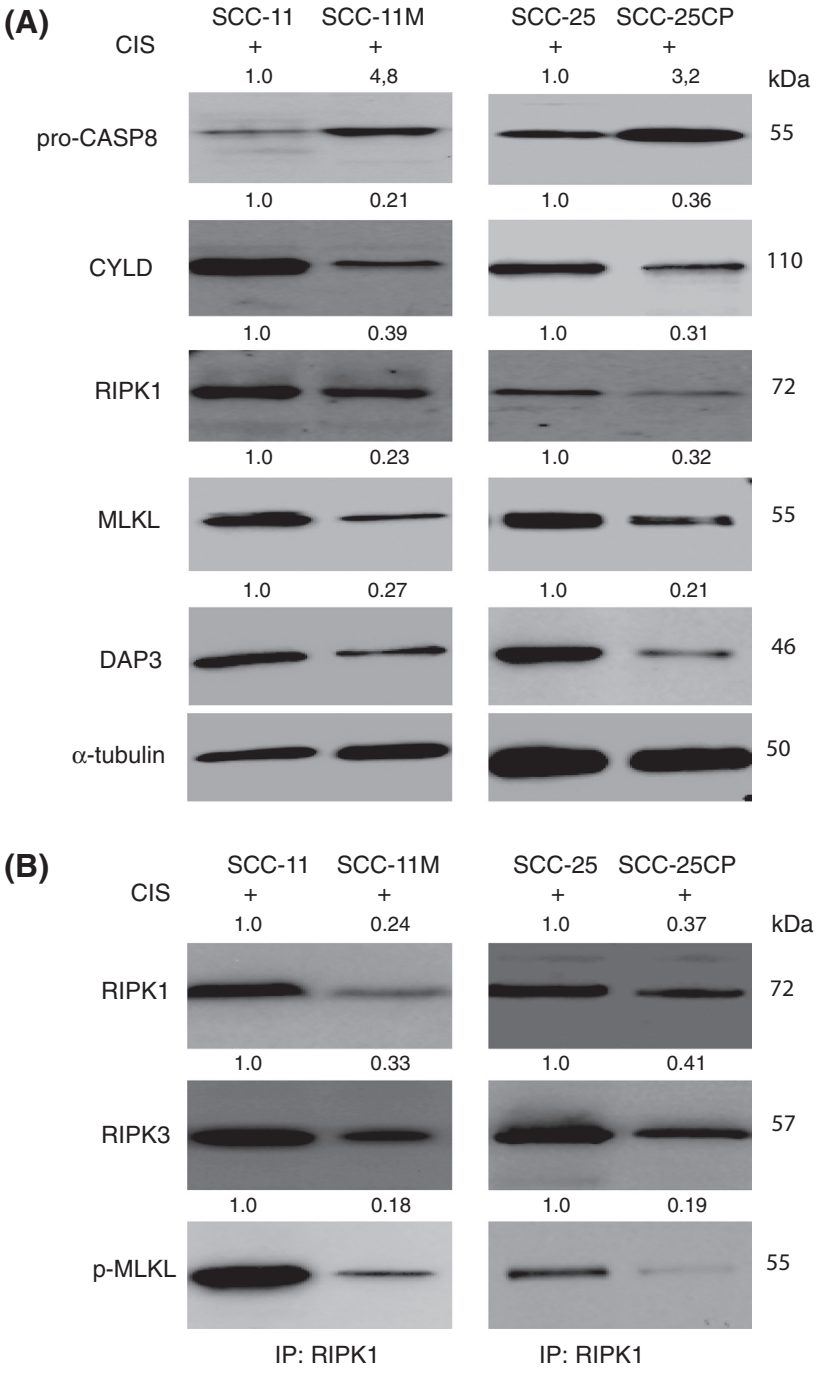

Fig. 3. Expression of protein targets in SCC cells upon cisplatin exposure. All SCC cells were exposed to 10 μg/ml cisplatin (CIS) for 16 h. (A, left panels) SCC-11 cells versus SCC-11M cells. (A, right panels). SCC-25 cells versus SCC-25CP cells. (A) Immunoblotting was performed with the indicated antibodies. Each blot was first probed with the indicated antibodies and after stripping with an antibody to α-tubulin used as a loading control. Lines between blots indicate the separate gel runs. Our data showed that the expression of pro-CASP8 was downregulated, while the expression of CYLD, RIPK1, MLKL, and DAP3 was upregulated in SCC-11 cells and SCC-25 cells compared to SCC-11M cells and SCC-25CP cells, respectively. (B) Co-immunoprecipitation of RIPK3, and p-MLKL with the antibody to RIPK1 showed the formation of RIPK1/RIPK3/p-MLKL protein complexes in SCC-11 cells and SCC-25 cells upon cisplatin exposure. Blots were scanned and quantified in triplicate. Values (shown above the blots) normalized by α-tubulin levels were expressed as a fold change to a scrambled control defined as 1.

Necroptosis is often induced by the ligand-activated TNFR1 through a RIPK1- and/or RIPK3-dependent pathway [2–6,22]. Other death receptors (e.g. CD95, TRAIL, etc.), as well as TNF-related weak inducer of apoptosis, lymphotoxin β, and ectodermal dysplasia receptor could also trigger this pathway [2–7,22]. Furthermore, different kinds of physical–chemical stress stimuli can initiate necroptosis, including reactive oxygen species, DNA damaging anticancer drugs, and ionizing radiation [2–6,23].

Binding of the TNFR1 by TNFα leads to recruitment of various proteins, such as tumor necrosis factor receptor type 1-associated death domain protein (TRADD), RIPK1, cIAP proteins, TRAF-2, and -5 into a multimeric protein complex called TNFR1 complex [2–7,24–26]. Within this complex, RIPK1 is polyubiquitinated by cIAP proteins leading to the activation of the NF-κB [27,28]. TNF receptor (TNFR) is then rapidly internalized upon ligand binding resulting in alterations in the posttranslational modifications of

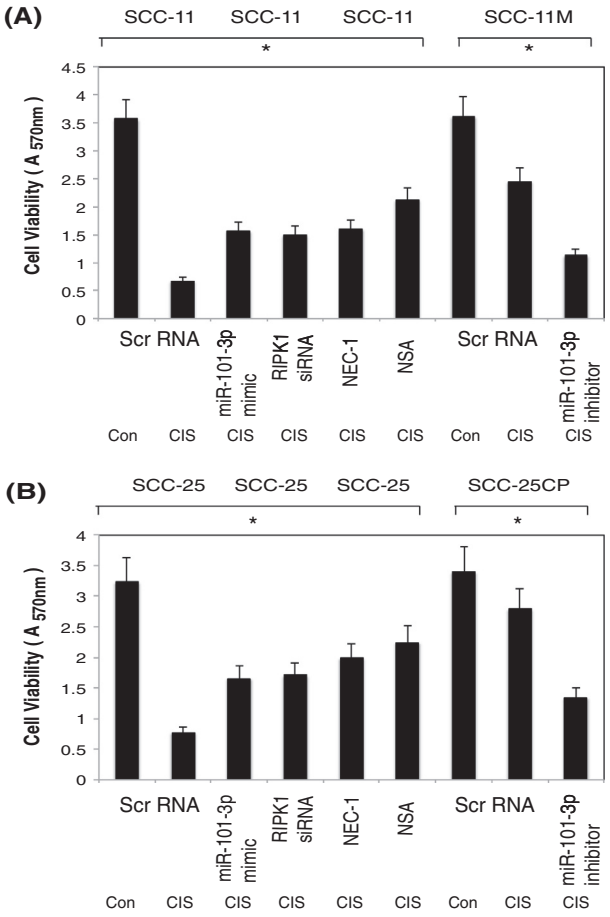

Fig. 4. Silencing of RIPK1 modulated the survival of SCC-11/11M cells and SCC-25/25CP cells upon cisplatin exposure. (A) SCC-11 cells cells were transfected with the scrambled (Scr) RNA, miR-101-3p mimic and siRNA against RIPK2. SCC-11M cells were transfected with the Scr RNA and miR-101-3p inhibitor. After 36 h post-transfection, cells were exposed to control media (Con) and 10 μg/ml cisplatin (CIS) for 16 h. SCC-11 cells were also treated with the RIPK1 inhibitor, necrostatine (NEC-1), and MLKL inhibitor, necrosulfonamide (NSA). (B) SCC-25 cells were transfected with the Scr RNA, miR-101-3p mimic and siRNA against RIPK2. SCC-25CP cells were transfected with the Scr RNA and miR-101-3p inhibitor. After 36 h post-transfection, cells were exposed to control media (Con) and 10 μg/ml cisplatin (CIS) for 16 h. SCC-25 cells were also treated with the RIPK1 inhibitor, necrostatine (NEC-1), and MLKL inhibitor, necrosulfonamide (NSA). All cells were also exposed to control media (Con) and 10 μg/ml cisplatin (CIS) for 16 h, as indicated. Cell viability assay. Assay was performed using five independent experiments in triplicate and compared to control conditions (*, $P < 0.05$).

TNFR-associated proteins [29]. RIPK1 becomes deubiquitinated by CYLD, which in turn increases the RIPK1 protein level [28,29]. When CASP8 is inhibited or downregulated, RIPK1 increases its ability to form a complex with RIPK3 triggering the necroptotic pathway [30]. This RIPK1/3 cytoplasmic protein complex "necrosome" involves the reciprocal phosphorylation of RIPK1 and RIPK3 resulting in MLKL phosphorylation [24–28]. The latter plays a decisive role in the transduction of the necroptotic signal to cell death [31,32].

A number of anti-cancer drugs have been shown to initiate necroptosis in cancer cells [33–38]. Shikonin, a naturally occurring naphthoquinone, has been reported inducing a necroptotic cell death in drug- and apoptosis-resistant cancer cells [34]. Staurosporine, an inhibitor of protein kinases, triggers necroptosis in leukemia cells when caspase activation is blocked, and the necroptotic pathway was inhibited by the RIPK1 inhibitor, NEC-1, and MLKL inhibitor, NSA [35]. Obatoclax, an inhibitor of anti-apoptotic BCL2 proteins, was shown to induce autophagy-dependent necroptosis in glucocorticoid-resistant acute lymphoblastic leukemia [37]. Obatoclax was also shown to trigger a physical interaction of ATG5 (a component of autophagosomal membranes) with the key regulators of the "necrosome", RIPK1 and RIPK3 [37].

Our previous reports have shown that the exposure of head and neck SCC-11 cells to cisplatin induced the phosphorylation of ΔNp63α by the ATM kinase [15]. However, the knock-in alteration of the ATM target site in the ΔNp63α protein (S385 to G385) diminished the ability for mutated ΔNp63α to be phosphorylated in SCC-11M cells [15]. We have found that the wild type ΔNp63α (p-ΔNp63α) and mutated ΔNp63α (non-p-ΔNp63α) differentially regulate the expression of specific microRNAs in SCC-11 cells and SCC-11M cells suggesting that the levels of these microRNA protein targets might be also affected [16]. We have observed that the ΔNp63α-responsive microRNAs are very likely to modulate the protein targets involved in cell cycle arrest, apoptosis, and autophagy, and even epigenetic regulation [16–18,20].

Since p63 was previously shown to transcriptionally activate the expression of several death domain receptors involved in TNF signaling [13], we explored a potential role for ΔNp63α in regulation of necroptotic pathway. We observed that a few ΔNp63α-responsive microRNAs might act as regulators of proteins implicated in the necroptotic signaling. We showed that the specific microRNAs (miR-485-5p, miR-221-3p, miR-101-3p, miR-22-3p, miR-155-5p, and miR-214-3p) modulated the luciferase activities driven by the CASP8, CYLD, RIPK1, MLKL, DAP3, and DAPK2 3′-UTRs in vitro. We further showed that the endogenous CYLD, RIPK1, MLKL, DAP3, and DAPK2 protein levels were higher in sensitive SCC cells (SCC-11, SCC-25) than in resistant SCC cells (SCC-11M, SCC-25CP). However, the CASP8 protein levels were lower in sensitive SCC cells than in resistant SCC cells. We next showed that the cisplatin exposure led to a varied formation of response in the RIPK1/RIPK3/p-MLKL complex in sensitive and resistant SCC cells. We observed a higher degree of the complex formation in sensitive SCC cells than in resistant SCC cells. We finally observed that the viability of SCC cells sensitive and resistant to cisplatin treatment could be potentially altered using the miR-101-3p mimic or inhibitor, as well as with RIPK1 siRNA, and necroptotic inhibitors. We found that the miR-101-3p mimic could increase the resistance of sensitive SCC cells to cisplatin, while the miR-101-3p inhibitor increased the sensitivity of resistant SCC cells to the drug. Overall, these data suggests a potential involvement of necroptotic pathway in SCC cell response to cisplatin exposure supporting the previous report showing necroptosis in cisplatin- and inhibitor of apoptosis (IAP) antagonist-resistant ovarian carcinomas [36].

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.04.020.

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